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(54) **SECRETORY SIGNAL GENE AND EXPRESSION VECTOR HAVING THE SAME**

GEN FÜR EIN SEKRETIONSSIGNAL UND DIESES ENTHALTENDER VEKTOR

GENE DE SIGNAL DE SECRETION ET VECTEUR D'EXPRESSION COMPRENANT CE SIGNAL

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The applicant has subsequently filed a sequence listing and declared, that it includes no new matter (0450).



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Description

## TECHNICAL FIELD

[0001] The present invention relates to a secretion signal gene having a base sequence encoding a polypeptide derived from the secretion signal of a precursor of a mating pheromone (P-factor), which concerns mating of fission yeast Schizosaccharomyces pombe (hereinafter referred to as S.pombe). The present invention also relates to an expression vector containing the secretion signal gene and a foreign of heterologous protein structural gene, and efficient secretory production of the heterologous protein structural gene product by S.pombe or the like.

## BACKGROUND ART

[0002] Hitherto, production of heterologous proteins utilizing genetic recombination technology has been extensively conducted by using microorganisms such as Escherichia coli, Saccharomyces cerevisiae or Bacillus, animal cells, plant cells and insect cells. As such heterologous proteins various biogenic proteins are considered to be accessible, and many of them have been industrially produced by using these living organisms for medical use so far.

[0003] However, methods employing procaryotes are not effective for all polypeptides, and it is not always easy to reproduce the complicated post-translational modification of eucaryotic proteins and to reproduce the natural steric structures. In addition, Escherichia coli has a characteristic endotoxin, which might contaminate end products. On the other hand, as for methods employing animal, plant or insect cells, these cells are more difficult to handle than microorganisms, their culture is costly, and production efficiency is low. For this reason, yeasts, eucaryotic microorganisms, are considered as the best for production of heterologous proteins, especially eucaryotic proteins. Their culture methods are well established, and they do not contain endotoxins. Therefore, expression vectors for use in various yeast hosts have been developed so far (Romanos, M.A. et al., Yeast 8, 423-488, 1992).

[0004] Among various yeasts, S.pombe is considered to be closer to higher animals in various properties such as cell cycle, chromosomal structure and RNA splicing than other yeasts inclusive of Saccharomyces cerevisiae. The post-translational modification such as acetylation, phosphorylation and glycosylation of proteins produced in S.pombe seems fairly similar to that in animal cells (Russell, P.R. and Nurse, P., Cell 45, 781-782, 1986; Kaufer, N.F. et al, Nature 318, 78-80, 1985; Chappell, T.G. and Warren, G., J. Cell. Biol. 109, 2693-2702, 1989). Therefore, use of S.pombe as a host for expression of a heterologous protein is expected to provide a gene product closer to its natural form, like that produced by animal cells. Since yeasts have a lot of commonness in their culture methods, knowledges about other yeasts can be easily applied to the yeast. Therefore, it is obviously advantageous to use S.pombe for production of a heterologous protein by using microbiological methods and the DNA recombination technique.

[0005] However, S.pombe is far behind Escherichia coli and Saccharomyces cerevisiae in studies on genetic recombination using them. Especially, with respect to gene expression in S.pombe, only a small number of studies have been reported (Japanese Unexamined Patent Publications Nos. 181397/1986, 283288/1990 and 63596/1992). This is because development of expression vectors, which have powerful promoters, are stable in S.pombe cells and are suitable and convenient for introduction of a gene, has been retarded. Recent development of vectors for the fission yeast with a high expressivity which contains an animal virus-derived promoter region eventually opened the way to mass production of heterologous proteins by S.pombe (Japanese Unexamined Patent Publications Nos. 15380/1993 and 163373/1995, which disclose inventions of the present inventors). This technique enabled many intracellular proteins to be produced easily and therefore is fairly useful.

[0006] Production of (heterologous) eucaryotic secretory proteins by yeasts scarcely succeeded so far because yeasts can hardly recognize inherent signal sequences of heterologous secretory proteins and therefore can not secrete the products from the cells into culture media. Further, at the time of purification, it was necessary that after cell rupture, the desired protein should be isolated from various coexistent cell components to avoid inactivation. Secretory production of a heterologous protein is not only preferable in view of the easiness of purification, but also advantageous in that the product is identical or fairly similar to its naturally occurring counterpart in steric structure, because the protein to be secreted enters the secretory pathway in the host cells and undergoes appropriate processings such as formation of disulfide bonds and glycosylation.

[0007] However, few signal sequences that effectively function in the fission yeast have been reported (Tokunaga, M. et al., Yeast 9, 379-387, 1993; Bröker, M. et al., B.B.A. 908, 203-213, 1987), no secretory expression vectors have been practically developed. On the other hand, the present inventors studied P-factor, which is a protein secreted by S.pombe from the cells and involved in mating as a mating pheromone. As a result, they found the fact that after conversion from its precursor by various enzymes in S.pombe, P-factor is secreted into a culture medium. They also determined the amino acid sequence and the gene of the P-factor precursor (Imai, Y. and Yamamoto, M., Gene & Dev. 8, 328-338; Japanese Unexamined Patent Publication No. 327481/1994). The amino acid sequence of the P-factor precursor is SEQ ID NO: 1 in the Sequence Listing given afterwards.

DISCLOSURE OF INVENTION

[0008] The present inventors focused on the above-mentioned P-factor and tried to develop a secretory expression vector by utilizing its secretory property. Though, it had been unknown which regions of the precursor function effectively as secretion signals, as a result of further studies, the present inventors found the N-terminal about 60 amino acids of the P-factor precursor functions effectively as secretion signals. As a result of more detail studies, they found the existence of a presequence which is to be cut off by signal peptidase, and a prosequence, which is to be cut off by proteases in the endoplasmic reticulum or the Golgi apparatus during processing. Accordingly, the secretion signal of the P-factor precursor can be shorter than the N-terminal about 60 amino acids.

[0009] The present invention provides a gene encoding for a polypeptide functional as a secretion signal in S.pombe. The polypeptide may be a polypeptide identical, equivalent or analogous to the secretion signal of the P-factor precursor or a longer polypeptide containing the polypeptide (but shorter than the P-factor precursor itself). The polypeptide may have at least one extra amino acid residue that the secretion signal of the P-factor precursor does not inherently have, or have at least one different amino acid residues substituted for at least one amino acid residue in the secretion signal of the P-factor precursor. Further, the polypeptide may lack at least one amino acid residue in the secretion signal of the P-factor precursor. Hereinafter, a polypeptide functional as a secretion signal in S.pombe is referred to simply as the secretion signal.

[0010] The present invention relates to a gene encoding the secretion signal (hereinafter referred to as secretion signal gene), a multicloning vector having the secretion signal gene, an expression vector having the secretion signal gene and a heterologous protein structural gene, a transformant from a non-human eucaryotic host which carries a recombinant DNA having the secretion signal gene or the expression vector, and a method of producing a heterologous protein by using the transformant. The present invention provides:

a secretion signal gene having a base sequence encoding a polypeptide functional as a secretion signal in S.pombe;
a secretion signal gene having a base sequence encoding a secretion signal of the P-factor precursor produced by S.pombe or a polypeptide containing the amino acid sequence of the secretion signal and functional as a secretion signal in S.pombe, in which addition, deletion or substitution of at least one amino acid residue have been made;
a secretion signal gene having a base sequence encoding a polypeptide having an amino acid sequence from the 1st amino acid to the 16 - 160th amino acid of SEQ ID NO: 1, wherein addition, deletion or substitution of at least one amino acid residue may have been made;
a secretion signal gene having a base sequence encoding a polypeptide having an amino acid sequence from the 1st amino acid to the 22±6th, the 31±6th or the 57±6th amino acid of SEQ ID NO: 1, wherein addition, deletion or substitution of at least one amino acid residue may have been made;
a secretion signal gene having a base sequence encoding the above polypeptide, wherein the polypeptide has at least one additional amino acid residue at the carboxy terminus so as to have a carboxy-terminal amino acid sequence of [-Lys-Lys-Arg];
a multicloning vector for construction of an expression vector for expression of a heterologous protein in a non-human eucaryotic host cell, which has the above-mentioned secretion signal gene upstream from a gene introduction site so that the secretion signal gene can be directly ligated with a heterologous protein structural gene to be introduced;
an expression vector to be expressed in a non-human eucaryotic host, which has a structural gene of a protein of a secretion signal and a heterologous protein bonded together in this order from the amino terminus, wherein the gene of the secretion signal is the above-mentioned secretion signal gene;
an isolated non-human eucaryotic host cell (hereinafter referred to as transformant), which carries a recombinant DNA containing a structural gene of a protein of the above secretion signal gene and a heterologous protein bonded together in this order from the amino terminus, or the above expression vector; and
a method of producing a heterologous protein, which comprises culturing the transformant so that the heterologous protein is produced and accumulated in the culture, and collecting it.

[0011] In a transformant having the secretion signal gene of the present invention, a heterologous protein synthesized with a secretion signal bonded is cut in the cells so as to shed the secretion signal, and is secreted from the cells. Therefore, it is possible to obtain a desired protein by isolating it from the culture medium, and to produce a desired protein effectively and easily.

[0012] According to the present inventors' studies, the longest secretion signal in Examples is a polypeptide segment of up to the 57th amino acid. However, in the present invention the secretion signal may be a longer polypeptide segment containing this polypeptide (in Example 1, a polypeptide of up to the 59th amino acid was used). In this case, a heterologous protein is secreted with amino acid residues in the polypeptide segment downstream from the cleaved site (hereinafter referred to as additional segment) bonded. The object of the present invention can be achieved, when the secreted

protein is useful or at least not adverse (for example, when the additional segment can be removed afterward) even if the intended heterologous protein is secreted with the additional segment bonded to it. Further, considering the sequence from the 55th amino acid to the 59th amino acid of the polypeptide segment, where the polypeptide segment is cleaved, the sequence of the first about 160 amino acid residues contains three more sequences similar to the sequence from the 55th to the 59th amino acid, and it is anticipated that the cleavage can occur also at these three sites.

[0013] However, in general, a long additional segment is likely to be adverse to the desired heterologous protein. Accordingly, it is preferred that the secreted protein contains no additional segment or a short additional segment. In this sense, an expression vector preferably is so constructed as to induce expression of a heterologous protein bonded to a sequence having an amino acid sequence of the first at most 160 amino acids of SEQ ID NO: 1 containing a secretion signal, more preferably to a sequence having the amino acid sequence (SEQ ID NO: 2), which corresponds to the first at most 59 amino acids of SEQ ID NO: 1. The results of Examples show that when the secretion signal has an amino acid sequence of up to the 59th amino acid, the secreted heterologous protein has the 58th and 59th amino acid residues of the secretion signal at the amino terminus. Therefore, in this case, it is anticipated that a polypeptide from the 1st amino acid to the 57th amino acid of SEQ ID NO: 1 (or, of SEQ ID NO: 2) is more suitable as a secretion signal.

[0014] Cleavage occurs behind from the 55th amino acid to the 57th amino acid sequence of [-Lys-Lys-Arg-]. Therefore, it is anticipated that this sequence is a kind of cleavage signal. This suggest that cleavage also can occur behind the sequences of [-Lys-Lys-Arg-] from the 89th amino acid to the 91st amino acid, from the 123rd amino acid to the 125th amino acid and from the 157th amino acid to the 159th amino acid.

[0015] Further, a detailed study on the secretion signal aimed at use of a shorter sequence revealed that the amino acid sequence from the amino terminus to the 22nd amino acid constitutes the presequence, and the amino acid sequence from the 23rd (or the 25th) amino acid to the 31st amino acid constitutes the prosequence. Both the presequence and the prosequence can lead a desired protein bonded directly behind them to secretion. Therefore they are also useful as secretion signals, even though they are shorter than a sequence of the first about 60 amino acids from the amino terminus. On the other hand, according to a theoretical prediction, (von Heijne, G. "A new method for predicting signal sequence cleavage sites", Nucleic Acids Research, 14, 4683-4690 (1986)), the 16th [-Ala] and the 18th [-Pro] are possible rear ends of secretion signals shorter than the sequence of up to the 22nd amino acid. Therefore it is anticipated that a polypeptide having an amino acid sequence from the 1st amino acid to the 16th amino acid and a polypeptide having an amino acid sequence from the 1st amino acid to the 18th amino acid also can function as secretion signals.

[0016] A more detailed study revealed that the polyamino acid segment of from the amino terminus to about the 60th amino acid is cleaved in a higher probability especially at the carboxy terminus of the 57th Arg than the presequence of up to the 22nd (or the 24th) amino acid and the prosequence of up to the 31st amino acid. Therefore, in the case of a secretion signal of a short sequence, attachment of the sequence of [-Lys-Lys-Arg-] of from the 55th amino acid to the 57th amino acid directly behind the presequence or the prosequence leads to easier cleavage and is expected to efficient secretion. Actually, it is demonstrated that attachment of [-Lys-Arg] directly behind the 31st [-Lys-] improves efficiency of secretly production.

[0017] From the above, it is thought that the P-factor precursor is cleaved just behind the 22nd (or the 24th), the 31st and the 57th amino acids, and the polypeptides having the sequences of from the fist amino acid to these amino acids can function as secretion signals in S.pombe. Further, it is anticipated that longer or shorter sequences can also function as secretion signals in S.pombe. Therefore, the secretion signals of the present invention are primarily identical to these secretion signals of the P-factor precursor. The secretion signals of the present invention are polypeptides obtained by adding some amino acid residues to the C-termini of these polypeptides (such as the above-mentioned polypeptides of from the 1st amino acid to the 59th amino acid and Secretion Signal P2 Sequence which will be described later in Examples) and polypeptides obtained by deleting some amino acid residues at the C-termini of these polypeptides (such as Secretion Signal P1 Sequence which will be described later in Examples). Polypeptides obtained by substituting some amino acid residues of these polypeptides with other amino acid residues (such as amino acid residues analogous to the amino acid residues to be substituted in properties) also can function as secretion signals.

[0018] More preferred secretion signals are (a) a polypeptide having a sequence from the 1st amino acid to the 22nd (or the 25th) amino acid, (b) a polypeptide having a sequence of from the 1st amino acid to the 31st amino acid, (c) a polypeptide having a sequence of from the 1st amino acid to the 57th amino acid, (d) polypeptides which have the C-termini ±6 residues (preferably ±3 residues) of the C-termini of the polypeptides (a) to (c), and (e) a polypeptides which correspond to polypeptide (a), (b) or (d) which has at least one additional amino acid residue so as to have the sequence of [Lys-Lys-Arg] at the C-terminus. In the case of (e), the number of additional amino acid residues is preferably at most 5.

[0019] The secretion signal gene of the present invention is a gene encoding the above-mentioned secretion signal. This secretion signal gene means genes corresponding to upstream regions of the gene of the P-factor precursor disclosed in Japanese Unexamined Patent Publication No. 327481/1994 and its modified versions. The secretion signal gene is obtainable both from a S.pombe chromosome and by synthesis. The secretion signal gene is by no means restricted to the sequences disclosed in the above-mentioned publication, and may be genes having sequences encoding the amino acid sequences described above.

[0020] A desired heterologous protein is expressed though an expression system containing its structural gene. As the expression system, a non-human eucaryotic cell transformed with an expression vector containing a heterologus protein structural gene is preferred. As the non-human eucaryotic cell, S.pombe is particularly preferred. In the expression system, the above-mentioned secretion signal gene is linked to the front end of the heterologous protein structural gene, and a protein of the secretion signal and the heterologous protein bonded together is produced. After intracellular processing, the heterologous protein is secreted out of the cell.

[0021] The multicloning vector of the present invention is a multicloning vector for construction of the expression vector which will be described below and enables expression of a heterologous protein in a non-human eucaryotic host cell. By introducing a heterologous protein structural gene into the multicloning vector, the expression vector described below can be constructed. The expression vector described below means those constructed by using the secretion signal-heterologous protein gene described below, and is not restricted to those constructed by using the multicloning vector of the present invention.

[0022] The multicloning vector of the present invention has the secretion signal gene described above upstream from a gene introduction site so that the secretion signal gene can be ligated directly with a heterologous protein structural gene to be introduced. When a heterologous protein structural gene is introduced into it to constitute an expression vector, the secretion signal gene and the heterologous protein structural gene are ligated and its expression of the ligation product produces a protein of the secretion signal and the heterologous protein bonded together.

[0023] The expression vector of the present invention has a gene corresponding to the above-mentioned secretion signal and a heterologous protein structural gene ligated together (hereinafter referred to as secretion signal-heterologous protein gene) and enables expression of the gene in a non-human eucaryotic host cell. This expression vector can be constructed by various methods by using the secretion signal-heterologous protein structural gene. For example, it can be constructed by introducing a secretion signal-heterologous protein gene at the multicloning site of a multicloning vector other than the multicloning vector of the present invention. It can be constructed without using a multicloning vector.

[0024] The expression vector of the present invention is preferably an expression vector having the structure disclosed in the above-mentioned Japanese Unexamined Patent Publication No. 15380/1993. It is preferred to construct the expression vector of the present invention by using the above-mentioned multicloning vector, which is disclosed in the specification of Japanese Patent Application No. 241581/1994, which relates to this expression vector or the method of constructing an expression vector using the multicloning vector. Nevertheless, the expression vector of the present invention may be an expression vector other than the expression vector disclosed in the above-mentioned publication, and may be of a chromosome integration type or of a type that can increase the copy number and can be present stably outside the nucleus. The expression vector of the present invention which has the structure disclosed in Japanese Unexamined Patent Publication No. 15380/1993 will be described below. However, the expression vector of the present invention is by no means restricted to it.

[0025] The expression vector of the present invention has a promoter region which controls the expression of the secretion signal-heterologous protein gene introduced. The promoter regulates the expression of the secretion signal-heterologous protein gene introduced downstream. The promoter is capable of functioning in a non-human eucaryotic cell and accelerates transcription of the introduced secretion signal-heterologous protein gene. Specifically, a promoter which can function in S.pombe cells is preferred.

[0026] As such a promoter, for example, alcohol dehydrogenase gene promoter, human cytomegalovirus gene promoter and human chorionic gonadotropin $\alpha$ gene promoter may be mentioned. Particularly preferred are promoters which strongly accelerate transcription, such as promoters from animal viruses (R. Toyama et al., FEBS Lett. 268, 217-221 (1990)). As such a preferable promoter, promoters from animal viruses, particularly human cytomegalovirus gene promoter may be mentioned.

[0027] The expression vector of the present invention may have a drug resistance gene such as an antibiotic resistance gene and other various genes. Further, it is also possible to make the vector of the present invention a shuttle vector by incorporating a promoter or a drug resistance gene capable of functioning in a procaryotic cell such as E. coli.

[0028] An expression vector must have a replication origin in order to be expressed in cells. However, for the expression vector of the present invention, it is not always necessary to have a replication origin. A replication origin can be introduced after the expression vector has been constructed. It is also possible to autonomously introduce a replication origin into an expression vector having no replication origin in a cell after the expression vector is taken up by the cell. These methods of introducing a replication origin are already known. For example, a vector having a replication origin capable of functioning in a yeast (hereinafter referred as a yeast vector) can be integrated with the expression vector of the present invention (Japanese Unexamined Patent Publication No. 15380/1993). It is also possible to let the expression vector of the present invention and a yeast vector fuse together in cells autonomously by introducing them into the same cells. Since these method of introducing a replication origin are available, it is not critical whether the expression vector of the present invention has a replication origin or not. However, in any case for expression of the expression vector in cells, it is necessary that the vector ultimately has a replication origin.

[0029] It is usually essential to introduce a drug resistance gene such as an antibiotic resistance gene into an expression

vector as a marker or for cloning. A gene which releases a leucine-requiring cell from the necessity for leucine (such as LEU2 gene) or a gene which releases a uracil-requiring cell from the necessity for uracil (such as URA3 gene) is often introduced. It is preferred also for the vector of the present invention to have an antibiotic resistance gene and a promoter which accelerates the transcription of the antibiotic resistance gene (hereinafter referred to as a second promoter). The second promoter is preferred to have a lower transcription accelerating activity than the promoter that accelerates the transcription of the above-mentioned secretion signal-heterologous protein gene. As the second promoter, promoters from animal viruses are preferred. Particularly preferred is SV40 early promoter. Although the antibiotic resistance gene regulated by the second promoter may be a conventional one, particularly in the present invention, neomycin resistance gene is preferred.

[0030] In the present invention, by the use of the expression vector having an antibiotic resistance gene, it is possible to increase the expression amount of the secretion signal-heterologous protein gene. For this purpose, the transcription accelerating activity of the second promoter must be lower than that of the promoter that regulates the secretion signal-heterologous protein gene. For the purpose of explanation, the case of culturing S.pombe that carries an expression vector having SV40 early promoter and neomycin resistance gene regulated by the promoter is given as an example. When the S.pombe transformant is cultured in a medium containing G418 (neomycin), the copy number of the expression vector in a cell increases with G418 concentration in the medium. Accordingly, by increasing the G418 concentration, it is possible to increase the copy number of the expression vector in a cell and thereby increase the expression amount of the secretion signal gene-heterologous protein. If the activity of the second promoter is higher than the promoter regulating the secretion signal-heterologous protein gene, there is no need to increase the copy number of the expression vector since a small copy number of the vector is enough to induce production of a sufficient amount of neomycin resistance protein (enzyme), and therefore it is impossible to increase the expression amount of the desired secretion signal heterologous protein.

[0031] The heterologous protein as the product of a protein structural gene is not particularly restricted, but is preferably a physiologically active protein of a higher animal. For example, a glycoproteins which are basically obtainable by secretory production by an animal cell and difficult to produce by using E.coli and proteins having complicated steric structures with many disulfide bonds, such as human serum albumin and interleukin-6, are particularly preferred.

[0032] The general technique of constructing the multicloning vector or the expression vector of the present invention is already known and disclosed, for example, in a reference, J.Sambrook et al., "Molecular Cloning 2nd ed.", Cold Spring Harbor Laboratory Press (1989). The multicloning vector and the expression vector of the present invention can be constructed by the above-mentioned method by using this conventional technique. As a strain of S.pombe to be used in the present invention as a host for the expression vector, ATCC 38399 (leu1-32h⁻) and ATCC 38436 (ura4-294h⁻) may, for example, be mentioned. These strains are available from American Type Culture Collection.

[0033] S.pombe can be transformed by using an expression vector by known methods, and a S.pombe transformant can be obtained by, for example, the lithium acetate method (K.Okazaki et al., Nucleic Acids Res., 18, 6485-6489 (1990)). The transformant is cultured in a known medium, and nutrient media such as YPD medium, minimal media such as MM medium (M.D. Rose et al., "Methods in Yeast Genetics", Cold Spring Harbor Laboratory Press (1990)) and the like may be used. The transformant is cultured usually at from 16 to 42°C, preferably at from 25 to 37°C, for from 8 to 168 hours, preferably for from 48 to 96 hours. Either of shaking culture and stationary culture can be employed, and, if necessary, the culture medium may be stirred or aerated.

[0034] As methods of isolating and purifying the protein produced in the culture, known methods, such as methods utilizing difference in solubility such as salting out and precipitation with a solvent, methods utilizing difference in molecular weight such as ultrafiltration and gel electrophoresis, methods utilizing difference in electric charge such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing difference in hydrophobicity such as reverse phase high performance liquid chromatography, and methods utilizing difference in isoelectric point such as isoelectric focusing may be mentioned.

[0035] The isolated and purified protein can be identified by conventional methods such as western blotting or assay of its activity. The structure of the purified protein can be defined by amino acid analysis, amino-terminal analysis, primary structure analysis and the like.

## BRIEF DESCRIPTION OF DRAWINGS

[0036] The accompanying drawings in association with the item best mode for carrying out the invention, are explained below.

[0037] Figure 1 illustrates the structure of the vector pSL26m, which was constructed in Example 1. Figure 2 and Figure 3 show SDS-PAGE and western blotting patterns which demonstrate expression of human interleukin-6 and its variants obtained in Examples 2, 5, 8, 11 and 14. Figure 4 illustrates the structure of the expression vector pSL2P06a'cl constructed in Example 4. Figure 5 illustrates the structure of the expression vector pSL2P16a'cl constructed in Example 7. Figure 6 illustrates the structure of the expression vector pSL2P26a'cl constructed in Example 10. Figure 7 illustrates

the structure of the expression vector pSL2P36'cl constructed in Example 13. Figure 8 illustrates the structure of the expression vector pSL2P3M1 constructed in Example 16.

## BEST MODE OF CARRYING OUT THE INVENTION

**[0038]** Now, the present invention will be described in further detail with reference to Examples. However, it should be understood that the technical scope of the present invention is by no means restricted to such specific examples.
**[0039]** Reference Examples 1 and 2 explain the methods of transforming and culturing a yeast employed in Examples.

### REFERENCE EXAMPLE 1: Transformation of a yeast

**[0040]** A leucine-requiring strain, S.pombe leul-32h$^-$ (ATCC38399) was used as the host. Host cells were grown in animal medium until the cell number became (0.5-1) $\times$ $10^7$ cells/ml. The cells were collected and suspended in 0.1 M lithium acetate (pH 5) at a cell number of 1 $\times$ $10^9$ cells/ml. Then, the cell suspension was incubated at 30°C for 60 minutes. 1 $\mu$g of a PstI fragment of a yeast vector pAL7 (ars, stb, LEU) (Nucleic Acid Research 18, 6485-6489) and 2 $\mu$g of an expression vector obtained in Examples were added to 100 $\mu$l of the cell suspension, and 290 $\mu$l of 50% (W/V) PEG4000 (polyethylene glycol with a molecular weight of 4,000) was added, and they were mixed enough. Then,the mixture was incubated at 30°C for 60 minutes and at 43°C for 15 minutes and was allowed to stand at room temperature for 10 minutes. After removal of PEG4000 by centrifugation, the cells were suspended in an appropriate amount of a medium, and the suspension was spread on a minimum medium. The transformation ratio was at least $10^5$ $\mu$g (pAL7).
**[0041]** An appropriate number of the transformants obtained above were harvested, and each transformant was suspended in 300 $\mu$l of water. A 3 $\mu$l portion of the suspension was spread on YEA medium (yeast extract 5g, glucose 30 g, agar 20 g/$\ell$ containing G418 (25 $\mu$g/ml) and three days after, the colonies formed were picked up for use.

### REFERENCE EXAMPLE 2: Culture of yeast

**[0042]** The transformed S.pombe (leu1-32h$^-$) strain was cultured in 5 $\mu$l of MM medium containing 1 wt% Casamino acid and 2 wt% glucose (Alfa et al. "Experiments with Fission Yeast" Cold Spring Harbor Laboratory Press 1993) in the presence of G418 (25 $\mu$g/ml) at 32°C overnight, and 5$\times$$10^7$ cells withdrawn from the culture medium were added to 50 ml of MM medium containing G418 (200 $\mu$g/ml), 1 wt% Casamino acid and 2 wt% glucose and cultured at 32°C for 48 hours. Then it was centrifuged to collect the culture medium.

### EXAMPLE 1

Preparation of human interleukin-6 secretory vector

**[0043]** PCR was performed by using a plasmid pAG9-8-1 (Japanese Unexamined Patent Publication No. 224097/1995) obtained by introducing the whole cDNA of human interleukin-6 into a commercially available vector pUC19 (sold by Boehringer Co., Ltd.) as the template and oligo DNAs shown in SEQ ID NOs:3 and 4 as primers to multiply the region containing the ORF (open reading frame) of mature interleukin-6. The fragment thus obtained was subjected to double digestion by restriction enzymes EcoRI (sold by Takara Shuzo Co.) and HindIII (sold by Takara Shuzo Co.) for terminal treatment. After agarose gel electrophoresis, the band corresponding to about 600 base pairs was excised, and a gene fragment insertion was isolated by the glass beads method by using DNA-PREP (sold by Asahi Glass Company Ltd.).
**[0044]** PCR was performed by using a plasmid pADMP2 (Japanese Unexamined Patent Publication No. 327481/1994) containing the whole map2 gene of S.pombe as the template, and oligo DNAs shown in SEQ ID NOs: 5 and 6 as primers to multiply the region containing P-factor secretion signal sequence. Double digestion by restriction enzymes BspHI (sold by New England Biolab Co.) and EcoRI for terminal treatment was followed acrylamide gel electrophoresis. The band corresponding to about 200 base pairs was excised, and a signal insertion fragment was eluted from the gel.
**[0045]** An expression vector pTL2M for S.pombe (Japanese Unexamined Patent Publication No. 163373/1995) constructed by using a vector pcD4B disclosed in Japanese Unexamined Patent Publication No. 15380/1993 was subjected to double digestion by restriction enzymes AflIII (sold by New England Biolab Co.) and HindIII, and then to agalose gel electrophoresis. The band corresponding to about 5,000 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP.
**[0046]** These three fragments were ligated by means of a DNA ligation kit (Takara Shuzo Co.). E.coli DH5 strain (sold by Toyobo Co.) was transformed and screened for possession of the intended plasmid constructed properly. Figure 1 illustrates the structure of the expression vector pSL26m thus obtained. pSL26m was prepared in a large amount by the alkali SDS method and identified by restriction map and partial base sequence analyzes as a plasmid having the intended sequence. The amino acid sequence of human interleukin-6 anticipated from the base sequence was SEQ ID NO: 7

and consisted of 185 residues. The amino acid sequence of the secretion signal sequence was SEQ ID NO: 2 and consisted of 59 residues.

EXAMPLE 2

Secretory production of human interleukin-6

[0047] Yeast was transformed with the secretory vector prepared in Example 1 in accordance with Reference Example 1 and then cultured in accordance with Reference Example 2. 50 ml of the culture medium was concentrated about 200 times by means of a membrane filter manufactured by Amicon Co., Ltd. The concentrated sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. Figure 2 shows the resulting SDS-PAGE pattern. In Figure 2, lane 1 is the supernatant of S.pombe/pSL2M (control) culture, and lane 2 is the supernatant of S.pombe/pSL26m culture.

[0048] As shown in Figure 2, several bands corresponding to molecular weights of at least 50,000 were observed, while in the low molecular weight region, a band at about 21 K and another band at a little smaller molecular weight were mainly detected.

[0049] The results of western blotting analysis of the supernatants were shown in Figure 3. In Figure 3, lane 1 is the supernatant of S.pombe/pSL2M (control) culture, cutting and lane 2 is the supernatant of S.pombe/pSL26m culture. The bands at 21 K and a little lower molecular weight were identified as attributed to human interleukin-6. Bands of little lower molecular weights seemed attributed to decomposition products.

EXAMPLE 3

Determination of the amino-terminal sequence of secreted protein

[0050] The protein isolated from the 21 K band obtained by the SDS-PAGE electrophoresis in Example 2 was analyzed from the amino terminus by a protein sequencer ("Shimadzu PSQ-1") and found to have an amino terminal sequence of Glu-Phe-Met-Pro-Val-Pro-Pro-. This indicates that it is secreted into the medium after accurate processing between Lys and Glu of the secretion signal. Similar investigation of the minor band of a lower molecular weight revealed an extra cleavage between the 9th Lys and the 10th Asp.

EXAMPLE 4

Preparation of secretory vector for interleukin-6 variant by using secretion signal

[0051] PCR was performed by using a plasmid pTL26a'Cl (Japanese Unexamined Patent Publication No. 224097/1995) containing the cDNA of a human interleukin-6 variant as the template and oligo DNAs represented by SEQ ID NOs: 8 and 9 to multiply a region containing the ORF of the interleukin-6 variant. The fragment thus obtained was subjected to double digestion by restriction enzymes EcoRI and HindIII for terminal treatment and then subjected to agalose gel electrophoresis. The band corresponding to about 600 base pairs was excised, and a gene insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0052] A plasmid pSL26m (Example 1) containing cDNA of P-factor secretion signal sequence of S.pombe was subjected to double digestion by restriction enzymes EcoRI and HindIII for terminal treatment and then subjected to agalose gel electrophoresis. The band corresponding to 5,000 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP.

[0053] The two fragments were ligated by means of a DNA ligation kit. After transformation of E.coli DH5 strain, the E.coli strain was screened for possession of the secretory vector pSL2P06a'Cl properly constructed as shown in Figure 4 through restriction map analysis. pSL2P06a'Cl was prepared in a large amount by the alkali-SDS method, and the base sequences of the ORF of the interleukin-6 variant and the region corresponding to the P-factor secretion signal sequence were determined. As a result, the amino acid sequence of the interleukin-6 variant was expected from the base sequence to be represented by SED ID NO: 10 and consist of 162 residues, and the secretion signal sequence, which was designated as secretion signal "P0" sequence, was made of 59 residues and had an amino acid sequence represented by SEQ ID NO: 2.

EXAMPLE 5

Secretory production of human interleukin-6 variant by using secretion signal P0 sequence.

[0054] Yeast was transformed with the secretory vector prepared in Example 4 in accordance with Reference Example 1 and then cultured in accordance with Reference Example 2. 50 ml of the culture medium was concentrated about 200 times by means of a membrane filter manufactured by Amicon Co., Ltd. The concentrated sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. Figure 2 is the resulting SDS-PAGE pattern. In Figure 2, lane 3 is the supernatant of S.pombe/pSL2P06a'CI culture

[0055] As shown in Figure 3, while several bonds were detected in the region of molecular weight of at least 50,000, in the region of lower molecular weight, only one band was detected at about 18 K.

[0056] The results of western blotting analysis of the supernatant of the culture are shown in Figure 3. Lane 3 in Figure 3 is the supernatant of the S.pombe/pSK2P06a'CI culture. The band at 18 K in lane 3 in Figure 3 was identified as attributed to a human interleukin-6 variant (IL-6a'CI).

EXAMPLE 6

Determination of the amino-terminal sequence of secreted protein

[0057] The protein extracted by a conventional method from the band at 18 K obtained by SDS-PAGE electrophoresis in Example 5 was analyzed from the amino terminus by a protein sequence ("Shimadzu PSQ-1") and found to have an amino terminal sequence of Glu-Phe-Pro-Val-Pro-Pro-Thr-Ser-Ser-Glu. This indicates that IL-6a'CI, which is a variant which lacks the 9th and the 10th Lys-Asp from the N terminus of interleukin-6, is secreted into the medium after accurate processing between Lys and Glu of the secretion signal. Therefore, the extra cleavage in Example 3 is unnecessary to secretion, and unless a specific sequence is present in the molecule, only a type of protein with a constant terminus is secreted.

EXAMPLE 7

Preparation of secretory vector for interleukin-6 variant by using secretion signal P1 sequence

[0058] PCR was performed by using a plasmid pSL2P06a'CI (Example 4) containing the cDNA of a human interleukin-6 variant as the template and oligo DNAs represented by SEQ ID NOs: 11 and 12 to multiply a region containing the ORF of the interleukin-6 variant. The fragment thus obtained was subjected to double digestion by restriction enzymes HaeII (sold by Takara Shuzo co.) and HindIII for terminal treatment and then subjected to agarose gel electrophoresis. The band corresponding to about 500 base pairs was excised, and a gene insertion fragment was isolated by the glass beads method by using DNA-PREP

[0059] PCR was performed by using a plasmid pSL2P06a'CI (Example 4) containing the cDNA of the P-factor secretion signal sequence of S.pombe as the template, and oligo DNAs represented by SEQ ID NOs: 13 and 14 as primers to multiply a region containing the P-factor secretion signal sequence. The fragment thus obtained was subjected to double digestion by restriction enzymes SpeI (sold by Takara Shuzo Co.) and HaeII for terminal treatment and then subjected to agarose gel electrophoresis. The band corresponding to about 700 base pairs was excised, and a signal insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0060] An expression vector pTL2M for S.pombe (Japanese Unexamined Patent Publication No. 163373/1995) was subjected to double digestion by restriction enzymes SpeI and HindIII for terminal treatment and subjected to agarose gel electrophoresis. The band corresponding to about 4,500 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP

[0061] These three fragments were ligated by means of a DNA ligation kit. After transformation of E.coli DH5 strain, E.coli clones were screened for possession of secretory vector pSL2P16a'CI properly constructed as shown in Figure 5 through restriction map analysis.

[0062] pSL2P16a'CI was prepared in a large amount by the alkali-SDS method and the base sequences of the ORF of the interleukin-6 variant and the region corresponding to the P-factor secretion signal sequence were determined. From the base sequences, it is anticipated that the interleukin-6 variant has an amino acid sequence represented by SEQ ID NO: 15 and made of 163 residues, and the secretion signal sequence, which is designated as secretion signal "P1" sequence, has an amino acid sequence represented by SEQ ID NO: 16 and made of 30 residues.

EXAMPLE 8

Secretory production of human interleukin-6 variant by using secretion signal P1 sequence

[0063] Yeast was transformed with the secretory vector prepared in Example 7 in accordance with Reference Example 1 and then cultured in accordance with Reference Example 2. 50 ml of the culture medium was concentrated about 200 times by means of a membrane filter manufactured by Amicon Co., Ltd. The concentrated sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. Figure 2 is the resulting SDS-PAGE pattern. In Figure 2, lane 4 is the supernatant of S.pombe/pSL2P16a'Cl culture

[0064] As shown in Figure 2, while several bands were detected in the region of molecular weight of at least 50,000, in the region of lower molecular weight, two major bands were detected at about 19 K.

[0065] The results of the western blotting analysis of the supernatant of the culture are shown in Figure 3. In Figure 3, lane 4 is the supernatant of the S.pombe/pSL2P16a'Cl culture. The two bands at about 19 K in lane 4 in Figure 3 were identified as attributed to a human interleukin-6 variant IL-6a'Cl.

EXAMPLE 9

Determination of the amino terminal sequence of the secreted protein

[0066] The amino terminal sequences of the proteins extracted by a conventional method from the two bands at about 19 K obtained by SDS-PAGE electrophoresis in Example 5 were analyzed by means of a protein sequencer, and found to be Asp-PRo-Gly-Val-Val-Ser-Val-Ser-Ala-Pro for the upper band and Gly-Val-Val-Ser-Val-Ser-Ala-Pro-Val-Pro for the lower band. The results indicate that two cleavage sites are present, and that secretion signal P1 was cut between the 22nd Ala and the 23rd Asp from the N-terminus and between Pro and Gly, which are two residues closer to the C-terminus. It is highly probable that the secretion signal P1 sequence has two signal peptidase cleavage sites.

EXAMPLE 10

Preparation of interleukin-6 variant secretory vector by using secretion signal P2 sequence

[0067] PCR was performed by using a plasmid pSL2P06a'Cl (Example 4) containing the cDNA of a human interleukin-6 variant as the template and oligo DNAs represented by SEQ ID NOs: 17 and 18 as primers to multiply the region containing the ORF of the interleukin-6 variant. The fragment thus obtained was subjected double digestion by restriction enzymes HaeII and HindIII for terminal treatment and then subjected to agarose gel electrophoresis. The band corresponding to about 500 base pairs was excised, and a gene insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0068] PCR was performed by using a plasmid pSL2P06a'Cl (Example 4) containing the cDNA of P-factor secretion signal sequence of S.pombe as the template and oligo DNAs represented by SEQ ID NOs: 19 and 20 as primers to multiply the region containing the P-factor secretion signal sequence. The fragment thus obtained was subjected to double digestion by restriction enzymes SpeI and HaeII for terminal treatment and then to agalose gel electrophoresis. The band corresponding to about 700 base pairs was excised, and a signal insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0069] An expression vector pTL2M for S.pombe (Japanese Unexamined Patent Publication No. 163373/1995) was subjected to double digestion by restriction enzymes SpeI and HindIII for terminal treatment and then to agalose gel electrophoresis. The band corresponding to about 4,500 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP.

[0070] These three fragments were ligated by means of a DNA ligation kit. After transformation of E.coli DH5 strain, E.coli clones were screened for possession of secretory vector pSL2P26a'Cl properly constructed as shown in Figure 6 by restriction map analysis.

[0071] pSL2P26a'Cl was prepared in a large amount by the alkali-SDS method, and the base sequences of the ORF of the interleukin-6 variant and the region corresponding to the P-factor secretion signal sequence were determined. From the base sequences, it is anticipated that the interleukin-6 variant has an amino acid sequence represented by SEQ ID NO: 15 and the secretion signal sequence, which is designated as secretion signal "P2" sequence, has an amino acid sequence represented by SEQ ID NO: 21 made of 31 residues.

EXAMPLE 11

Secretory production of human interleukin-6 variant by using secretion signal P2 sequence

[0072] Yeast was transformed with the secretory vector prepared in Example 10 in accordance with Reference Example 1 and then cultured in accordance with Reference Example 2. 50 ml of the culture medium was concentrated about 200 times by means of a membrane filter manufactured by Amicon Co., Ltd. The concentrated sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by h Coomassie Brilliant Blue staining. Figure 2 shows the resulting SDS-PAGE pattern. In Figure 2, lane 5 is the supernatant of S.pombe/pSL2P26a'Cl culture.

[0073] As shown in Figure 2, while several bands were detected in the region of molecular weight of at least 50,000, in the region of lower molecular weight, two major bands and one main band were detected at about 19 K and at about 18.5 K, respectively.

[0074] The results of the western blotting analysis of the supernatant of the culture are shown in Figure 3. In Figure 3, lane 5 is the supernatant of the S.pombe/pSL2P26a'Cl culture. The two bands at about 19 K and the band that about 18.5 K in lane 5 in Figure 3 were identified as attributed to human interleukin-6 variant IL-6a'Cl.

EXAMPLE 12

Determination of the amino terminal sequence of the secreted protein

[0075] The amino terminal sequences of the proteins extracted by a conventional method from the two bands at about 19 K obtained by SDS-PAGE electrophoresis in Example 11 were analyzed by means of a protein sequencer, and found to be Asp-PRo-Gly-Val-Val-Ser-Val-Ser-Ala-Pro for the upper band and Gly-Val-Val-Ser-Val-Ser-Ala-Pro-Val-Pro for the lower band. The results indicate that two cleavage sites are present, and that secretion signal P1 was cut between the 22nd Ala and the 23rd Asp from the N-terminus and between Pro and Gly, which are two residues closer to the C-terminus. It is highly probable that the secretion signal has two signal peptidase cleavage sites. Further, the amino terminal sequence of the peptide extracted from the band at about 18.5 K was analyzed from the N-terminus and found to be Ser-Ala-Pro-Val-Pro-Pro-Thr-Ser-Ser-Glu. This indicates that the secretion signal P2 is cut between the 31st Lys and the 32nd Ser from the N-terminus during processing before secretion.

EXAMPLE 13

Preparation of interleukin-6 variant secretory vector by using secretion signal P3 sequence

[0076] PCR was performed by using a plasmid pSL2P06a'Cl (Example 4) containing the cDNA of a human interleukin-6 variant as the template and oligo DNAs represented by SEQ ID NOs: 22 and 23 as primers to multiply a region containing the ORF of the interleukin-6 variant. The fragment thus obtained was subjected double digestion by restriction enzymes AflII (sold by Nippon Gene Co.) and HindIII for terminal treatment and then subjected to agarose gel electrophoresis. The band corresponding to about 500 base pairs was excised, and a gene insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0077] PCR was performed by using a plasmid pSL2P06a'Cl (Example 4) containing the P-factor secretion signal sequence of S.pombe as the template and oligo DNAs represented by SEQ ID NOs: 24 and 25 as primers to multiply a region containing the P-factor secretion signal sequence. The fragment thus obtained was subjected to double digestion by restriction enzymes SpeI and AflII for terminal treatment and then to agalose gel electrophoresis. The band corresponding to about 700 base pairs was excised, and a signal insertion fragment was isolated by the glass beads method by using DNA-PREP.

[0078] An expression vector pTL2M for S.pombe (Japanese Unexamined Patent Publication No. 163373/1995) was subjected to double digestion by restriction enzymes SpeI and HindIII for terminal treatment and then to agalose gel electrophoresis. The band corresponding to about 4,500 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP.

[0079] These three fragments were ligated by means of a DNA ligation kit. After transformation of E.coli DH5 strain, E.coli clones were screened for possession of secretory vector pSL2P36a'Cl properly constructed as shown in Figure 6 through restriction map analysis.

[0080] pSL2P36a'Cl was prepared in a large amount by the alkali-SDS method, and the base sequences of the ORF of the interleukin-6 variant and the region corresponding to the P-factor secretion signal sequence were determined. From the base sequences, it is anticipated that the interleukin-6 variant has an amino acid sequence represented by SEQ ID NO: 15 and the secretion signal sequence, which is designated as secretion signal "P3" sequence, has an amino acid sequence represented by SEQ ID NO: 26 made of 34 residues.

EXAMPLE 14

Secretory production of human interleukin-6 variant by using secretion signal P3 sequence

**[0081]** Yeast was transformed with the secretory vector prepared in Example 13 in accordance with Reference Example 1 and then cultured in accordance with Reference Example 2. 50 ml of the culture medium was concentrated about 200 times by means of a membrane filter manufactured by Amicon Co., Ltd. The concentrated sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by the Coomassie Brilliant Blue staining.
Figure 2 is the resulting SDS-PAGE pattern. In Figure 2, lane 6 is the supernatant of S.pombe/pSL2P36a'Cl culture.

**[0082]** As shown in Figure 2, while several bands were detected in the region of molecular weight of at least 50,000, in the region of lower molecular weight, one band was detected at about 18 K.

**[0083]** The results of the western blotting analysis of the supernatant of the culture are shown in Figure 3. The band at about 18 K in lane 6 in Figure 3 was identified as attributed to IL-6a'Cl.

EXAMPLE 15

Determination of the amino-terminal sequence of the secreted protein

**[0084]** The N-terminal sequence of the protein isolated by a conventional method from the band at 20 K obtained by the SDS-PAGE electrophoresis in Example 14 was analyzed by a protein sequencer and found to be Ala-Pro-Val-Pro-Pro-Thr-Ser-Ser-Glu-. This indicates that the protein is secreted into the medium after accurate processing between Lys at the terminus of P3 signal and Ala at the N-terminus of Ll-6a'Cl.

EXAMPLE 16

Preparation of general-purpose secretory vector

**[0085]** PCR was performed by using an expression vector pTL2M for S.pombe (Japanese Unexamined Patent Publication No. 163373/1995) as the template and oligo DNAs represented by the SEQ ID NOs: 27 and 28 as primers to multiply the region containing a MCS (multicloning site) sequence. The fragment thus obtained was subjected to double digestion by restriction enzymes AflII and BglII (sold by Takara Shuzo Co.) for terminal treatment and then to agarose gel electrophoresis. The band corresponding to about 300 base pairs was excised, and a MCS insertion fragment was isolated by the glass beads method by using DNA-PREP.

**[0086]** A human interleukin-6 variant secretory vector pSL2P36a'Cl (Example 13) was subjected to double digestion by restriction enzymes AflII and BamHI (sold by Takara Shuzo Co.) for terminal treatment and then to agalose gel electrophoresis. The band corresponding to about 4,500 base pairs was excised, and a vector fragment was isolated by the glass beads method by using DNA-PREP.

**[0087]** These two fragments were ligated by means of a DNA ligation kit. After transformation of E.coli DH5 strain, E.coli clones were screened for possession of a secretory vector pSL2P3M1 constructed properly as shown in Figure 8.

**[0088]** pSL2P3M1 was prepared in a large amount by the alkali-SDS method, and the base sequences of the MCS sequence and the region corresponding to the P-factor secretion signal sequence were determined. As a result, the MCS sequence has a base sequence of 75 bp represented by SEQ ID NO: 29, and it is anticipated from the base sequence that the amino acid sequence of the secretion signal sequence is represented by the SEQ ID NO: 26.

Sequence Listing

**[0089]**

SEQ ID NO: 1
SEQUENCE LENGTH: 201 amino acids
SEQUENCE TYPE: amino acid
TOPOLOGY: linear
MOLECULAR TYPE: protein

Sequence

Met Lys Ile Thr Ala Val Ile Ala Leu Leu Phe Ser Leu Ala Ala Ala
1 5 10 15
Ser Pro Ile Pro Val Ala Asp Pro Gly Val Val Ser Val Ser Lys Ser
20 25 30
Tyr Ala Asp Phe Leu Arg Val Tyr Gln Ser Trp Asn Thr Phe Ala Asn
35 45
Pro Asp Arg Pro Asn Leu Lys Lys Arg Glu Phe Glu Ala Ala Pro Ala
50 55 60
Lys Thr Tyr Ala Asp Phe Leu Arg Ala Tyr Gln Ser Trp Asn Thr Phe
65 70 75 80
Val Asn Pro Asp Arg Pro Asn Leu Lys Lys Arg Glu Phe Glu Ala Ala
81 85 90 95
Pro Glu Lys Ser Tyr Ala Asp Phe Leu Arg Ala Tyr His Ser Trp Asn
100 105 110
Thr Phe Val Asn Pro Asp Arg Pro Asn Leu Lys Lys Arg Glu Phe Glu
115 120 125
Ala Ala Pro Ala Lys Thr Tyr Ala Asp Phe Leu Arg Ala Tyr Gln Ser
130 135 140
Trp Asn Thr Phe Val Asn Pro Asp Arg Pro Asn Leu Lys Lys Arg Thr
145 150 155 160

Glu Glu Asp Glu Glu Asn Glu Glu Glu Asp Glu Glu Tyr Tyr Arg Phe
165 170 175
Leu Gln Phe Tyr Ile Met Thr Val Pro Glu Asn Ser Thr Ile Thr Asp
180 185 190
Val Asn Ile Thr Ala Lys Phe Glu Ser
195 200 201

SEQ ID NO: 2

SEQUENCE LENGTH: 59 amino acids
SEQUENCE TYPE: amino acid
TOPOLOGY: linear
MOLECULAR TYPE: peptide

Sequence

```
Met Lys Ile Thr Ala Val Ile Ala Leu Leu Phe Ser Leu Ala Ala Ala
 1               5                  10                  15
Ser Pro Ile Pro Val Ala Asp Pro Gly Val Val Ser Val Ser Lys Ser
            20                  25                  30
Tyr Ala Asp Phe Leu Arg Val Tyr Gln Ser Trp Asn Thr Phe Ala Asn
        35                  40                  45
Pro Asp Arg Pro Asn Leu Lys Lys Arg Glu Phe
        50              55              59
```

SEQ ID NO: 3
SEQUENCE LENGTH: 30 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
GAATTCATGC CAGTACCCCC AGGAGAAGAT

SEQ ID NO: 4
SEQUENCE LENGTH: 31 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAGCTTATTA CATTTGCCGA AGAGCCCTCA G

SEQ ID NO: 5
SEQUENCE LENGTH: 25 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CGGTCATGAA GATCACCGCT GTCAT

SEQ ID NO: 6
SEQUENCE LENGTH: 27 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence

GGGAAGCTTA GCTCTCAAAT TTGGCAG

SEQ ID NO. 7
SEQUENCE LENGTH: 185 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS:single
TOPOLOGY: linear
MOLECULAR TYPE: peptide

```
Sequence
Met Pro Val Pro Pro Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His
 1               5                  10                  15
Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr
            20                  25                  30
Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser
        35                                  45
Asn Met Cys Glu Ser Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn
    50                  55                  60
Leu Pro Lys Met Ala Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn
65                  70                  75                  80
Glu Glu Thr Cys Leu Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu
                85                  90                  95
Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln
            100                 105                 110
Ala Arg Ala Val Gln Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln
        115                 120                 125
Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr
    130                 135                 140
Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln
145                 150                 155                 160
Asp Met Thr Thr His Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln
                165                 170                 175
Ser Ser Leu Arg Ala Leu Arg Gln Met
            180                 185
```

SEQ ID NO: 8
SEQUENCE LENGTH: 30 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAAGAATTCC CAGTACCCCC AACCTCTTCA

SEQ ID NO: 9
SEQUENCE LENGTH: 31 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAGCTTATTA CATTTGCCGA AGAGCCCTCA G

SEQ ID NO: 10
SEQUENCE LENGTH: 162 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: protein

Sequence

```
Pro Val Pro Pro Thr Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr
 1               5                  10                  15
Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Glu Ser Ser Lys
            20                  25                  30
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Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys
35 45
Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys
50 55 60
Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln
65 70 75 80
Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser
85 90 95
Thr Lys Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp
100 105 110
Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys
115 120 125
Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile
130 135 140
Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg
145 150 155 160
Gln Met
162

SEQ ID NO: 11
SEQUENCE LENGTH: 29 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CTTGGCGCCC CAGTACCCCC AACCTCTTC

SEQ ID NO: 12
SEQUENCE LENGTH: 10 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAAATGATTT AAAGGCTATA

SEQ ID NO: 13

SEQUENCE LENGTH: 20 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
TTGACTAGTT ATTAATAGTA

SEQ ID NO: 14
SEQUENCE LENGTH: 26 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CCAAGCGCTA ACTGAAACCA CACCAG

SEQ ID NO: 15
SEQUENCE LENGTH: 163 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: peptide

Sequence

Ala Pro Val Pro Pro Thr Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg
1 5 10 15
Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Glu Ser Ser
20 25 30
Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu
35 45
Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val
50 55 60
Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu
65 70 75 80
Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met
85 90 95
Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu
100 105 110
Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr
115 120 125
Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu
130 135 140
Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu
145 150 155 160
Arg Gln Met
163

SEQ ID NO: 16
SEQUENCE LENGTH: 30 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: Peptide

Sequence

Met Lys Ile Thr Ala Val Ile Ala Leu Leu Phe Ser Leu Ala Ala Ala
1 5 10 15

Ser Pro Ile Pro Val Ala Asp Pro Gly Val Val Ser Val Ser
20 25 30

SEQ ID NO: 17
SEQUENCE LENGTH: 29 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CTTGGCGCCC CAGTACCGCC AACCTCTTC

SEQ ID NO: 18
SEQUENCE LENGTH: 20 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAAATGATTT AAAGGCTATA

SEQ ID NO: 19
SEQUENCE LENGTH: 20 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
TTGACTAGTT ATTAATAGTA

SEQ ID NO: 20
SEQUENCE LENGTH: 29 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CCAAGCGCTC TTGCTAACTG AAACCACAC

SEQ ID NO: 21
SEQUENCE LENGTH: 32 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: peptide

Sequence

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Met | Lys | Ile | Thr | Ala | Val | Ile | Ala | Leu | Leu | Phe | Ser | Leu | Ala | Ala | Ala |
| 1 | | | | 5 | | | | | 10 | | | | | 15 | |
| Ser | Pro | Ile | Pro | Val | Ala | Asp | Pro | Gly | Val | Val | Ser | Val | Ser | Lys | Ser |
| | | | 20 | | | | | 25 | | | | | 30 | | 32 |

SEQ ID NO: 22
SEQUENCE LENGTH: 35 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CCTCTTAAGA AGCGTCCAGT ACCCCCAACC TCTTC

SEQ ID NO: 23
SEQUENCE LENGTH: 20 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY. linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAAATGATTT AAAGGCTATA

SEQ ID NO: 24
SEQUENCE LENGTH: 20 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY. linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
TTGACTAGTT ATTAATAGTA

SEQ ID NO: 25
SEQUENCE LENGTH: 29 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
CTTCTTAAGG CTAACTGAAA CCACACCAG

SEQ ID NO: 26
SEQUENCE LENGTH: 34 amino acids
SEQUENCE TYPE: amino acid
STRANDNESS: single
TOPOLOGY. linear
MOLECULAR TYPE: peptide

Sequence

Met Lys Ile Thr Ala Val Ile Ala Leu Leu Phe Ser Leu Ala Ala Ala
1 5 10 15

Ser Pro Ile Pro Val Ala Asp Pro Gly Val Val Ser Val Ser Leu Lys
20 25 30

Lys Arg
34

SEQ ID NO: 27
SEQUENCE LENGTH: 35 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
TTTCTTAAGA AGCGTACATG TGAATTCGAG CTCGG

SEQ ID NO: 28
SEQUENCE LENGTH: 36 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence
AAAAGATCTG ATATCGTCTT GTGACGTCAT TTTATT

SEQ ID NO: 29
SEQUENCE LENGTH: 75 base pairs
SEQUENCE TYPE: nucleic acid
STRANDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: other nucleic acid synthetic DNA
Sequence

CTTAAGAAGC GTACATGTGA ATTCGAGCTC GGTACCCGGG GATCCTCTAG AGTCGACCTG 60
CAGGCATGCA AGCTT 75

## Claims

1. Secretion signal gene encoding a polypeptide functional as a secretion signal in *Schizosaccharomyces pombe*, wherein the polypeptide consists of an amino acid sequence from the 1 st amino acid residue to the 31 ~ 6th amino acid residue of SEQ ID NO: 1 and 1 to 5 additional amino acid residues so as to have the sequence [-Lys-Lys-Arg] at the C-terminus of said polypeptide.

2. The secretion signal gene according to claim 1, wherein the polypeptide has the amino acid sequence of SEQ ID

NO: 26.

3. A multicloning vector for construction of an expression vector for expression of a protein in a eukaryotic host cell, which has the secretion signal gene according to claim 1 or 2 upstream from a gene introduction site so that the secretion signal gene can be directly ligated to the gene to be introduced.

4. An expression vector to be expressed in a eukaryotic host cell, which has a structural gene encoding a protein consisting of a secretion signal and a heterologous protein bonded together in this order from the amino terminus wherein the gene encoding the secretion signal is the secretion signal gene according to claim 1 or 2.

5. The expression vector according to claim 4, which has an animal virus-derived promoter region regulating the expression of the structural gene.

6. The expression vector according to one of claims 4 or 5, which has a neomycin resistance gene and a second animal virus-derived promoter regulating the expression of the neomycin resistance gene.

7. An isolated non-human eukaryotic host cell comprising a recombinant DNA containing a structural gene encoding a protein consisting of a secretion signal as encoded by the secretion signal gene according to claims 1 or 2 and a heterologous protein bonded together in this order from the amino terminus, or comprising the expression vector according to one of claims 4 or 5.

8. The host cell of claim 7, which is *Schizosaccharomyces pombe*.

9. A method of producing a heterologous protein, which comprises culturing the transformant of claim 7 or 8 so that the heterologous protein is produced and accumulated in the culture, and collecting said heterologous protein.

**Patentansprüche**

1. Sekretionssignal-Gen, kodierend ein Polypeptid, das als Sekretionssignal in *Schizosaccharomyces pombe* funktionell ist, worin das Polypeptid aus einer Aminosäure-Sequenz vom ersten Aminosäure-Rest bis zum 31. ± 6. Aminosäure-Rest der SEQ ID NO: 1 und 1 bis 5 zusätzlichen Aminosäure-Resten besteht, um am C-Terminus des Polypeptids die Sequenz [-Lys-Lys-Arg] aufzuweisen.

2. Sekretionssignal-Gen nach Anspruch 1, worin das Polypeptid die Aminosäure-Sequenz von SEQ ID NO: 26 aufweist.

3. Multiklonierender Vektor zur Konstruktion eines Expressionsvektors zur Expression eines Proteins in einer eukaryontischen Wirtszelle, der das Sekretionssignal-Gen nach Anspruch 1 oder 2 stromauf von einer Gen-Einführungsposition aufweist, wodurch das Sekretionssignal-Gen direkt an das einzuführende Gen geknüpft werden kann.

4. Expressionsvektor zur Expression in einer eukaryontischen Wirtszelle, der ein Strukturgen aufweist, das ein Protein kodiert, das aus einem Sekretionssignal und einem heterologen Protein, in dieser Reihenfolge vom Amino-Terminus zusammengebunden, besteht, worin das das Sekretionssignal kodierende Gen das Sekretionssignal-Gen nach Anspruch 1 oder 2 ist.

5. Expressionsvektor nach Anspruch 4, der eine Tiervirus-abgeleitete Promotorregion aufweist, die die Expression des Strukturgens reguliert.

6. Expressionsvektor nach einem der Ansprüche 4 oder 5, der ein Neomycin-Resistenzgen und einen zweiten Tiervirus-abgeleiteten Promotor, der die Expression des Neomycin-Resistenzgens reguliert, aufweist.

7. Isolierte nicht-menschliche eukaryontische Wirtszelle, umfassend eine rekombinante DNA, die ein ein Protein kodierendes Strukturgen enthält, das aus einem durch das Sekretionssignal-Gen nach einem der Ansprüche 1 oder 2 kodierten Sekretionssignal und einem heterologen Protein, in dieser Reihenfolge vom Amino-Terminus zusammengebunden, besteht, oder den Expressionsvektor nach einem der Ansprüche 4 oder 5 umfasst.

8. Wirtszelle nach Anspruch 7, die *Schizosaccharomyces pombe* ist.

9. Verfahren zur Herstellung eines heterologen Proteins, das das Kultivieren des Transformanten nach Anspruch 7 oder 8 aufweist, damit das heterologe Protein in der Kultur produziert und angehäuft wird, und Gewinnen des heterologen Proteins.

## Revendications

1. Gène signal de sécrétion codant un polypeptide fonctionnel en tant que signal de sécrétion dans *Schizosaccharomyces pombe*, dans lequel le polypeptide est constitué d'une séquence d'acides aminés allant du 1er résidu d'acide aminé au 31 :: 6ème résidu d'acide aminé de la SEQ ID NO : 1 et de 1 à 5 résidus d'acide aminé additionnels de façon à avoir la séquence [-Lys-Lys-Arg] à l'extrémité C dudit polypeptide.

2. Gène signal de sécrétion selon la revendication 1, dans lequel le polypeptide a la séquence d'acides aminés de la SEQ ID NO : 26.

3. Vecteur de multiclonage pour la construction d'un vecteur d'expression pour l'expression d'une protéine dans une cellule hôte eucaryote, qui a le gène signal de sécrétion selon la revendication 1 ou 2 en amont d'un site d'introduction de gène de façon que le gène signal de sécrétion puisse être directement ligaturé au gène devant être introduit.

4. Vecteur d'expression à exprimer dans une cellule hôte eucaryote, qui a un gène structural codant une protéine constituée d'un signal de sécrétion et d'une protéine hétérologue liés ensemble dans cet ordre à partir de l'extrémité amino, dans lequel le gène codant le signal de sécrétion est le gène signal de sécrétion selon la revendication 1 ou 2.

5. Vecteur d'expression selon la revendication 4, qui a une région de promoteur issu d'un virus animal régulant l'expression du gène structural.

6. Vecteur d'expression selon l'une des revendications 4 et 5, qui a un gène de résistance à la néomycine et un deuxième promoteur issu d'un virus animal régulant l'expression du gène de résistance à la néomycine.

7. Cellule hôte eucaryote non humaine isolée comprenant un ADN recombiné contenant un gène structural codant une protéine constituée d'un signal de sécrétion tel que codé par le gène signal de sécrétion selon la revendication 1 ou 2 et une protéine hétérologue, liés ensemble dans cet ordre à partir de l'extrémité amino, ou comprenant le vecteur d'expression selon l'une des revendications 4 et 5.

8. Cellule hôte selon la revendication 7, qui est *Schizosaccharomyces pombe*.

9. Procédé pour produire une protéine hétérologue, qui comprend la culture du transformé de la revendication 7 ou 8, de façon que la protéine hétérologue soit produite et accumulée dans la culture, et la collecte de ladite protéine hétérologue.

FIGURE 1

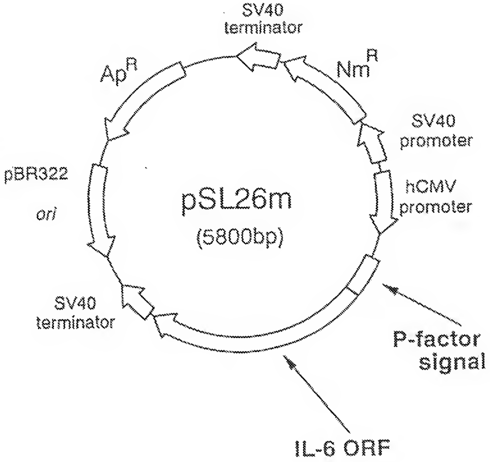

SV40
terminator
$Ap^R$
$Nm^R$
SV40
promoter
pBR322
*ori*
pSL26m
(5800bp)
hCMV
promoter
SV40
terminator
P-factor
signal
IL-6 ORF

FIGURE 2

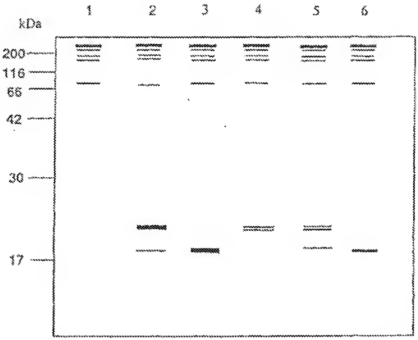



FIGURE 3

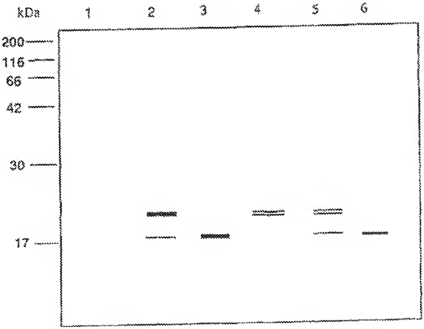


FIGURE 4

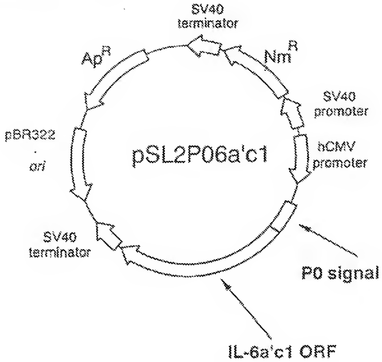



FIGURE 5

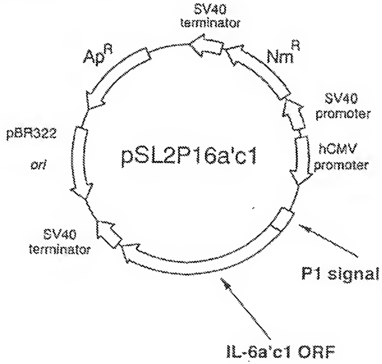


FIGURE 6

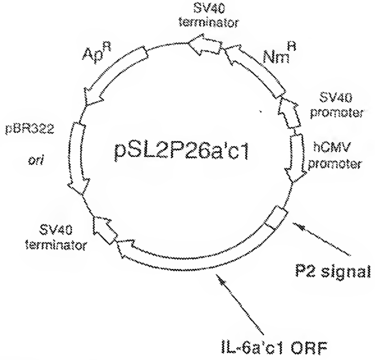



FIGURE 7

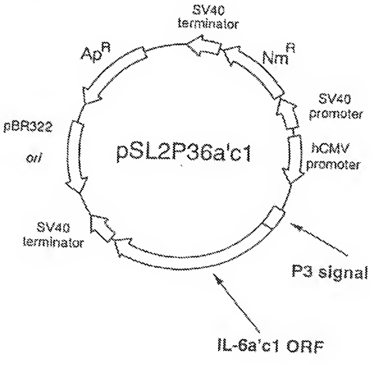
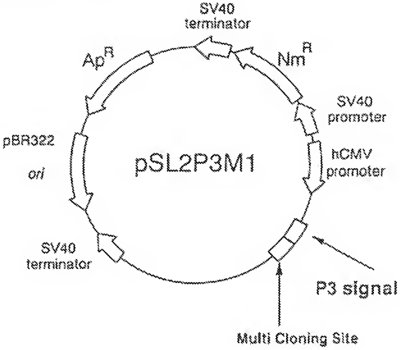


FIGURE 8


SV40
terminator
Ap$^R$
Nm$^R$
SV40
promoter
pBR322
ori
pSL2P3M1
hCMV
promoter
SV40
terminator
P3 signal
Multi Cloning Site